REMARKS

Applicant respectfully request entry of the foregoing and continued examination of the subject matter identified in caption, as amended, pursuant to and consistent with 37 C.F.R. § 1.114, and in light of the remarks which follow.

Claims 1-16 are pending. Claims 3, 4, and 6-13 have been withdrawn from consideration. Applicants reserve the right to file at least one continuation application directed to any subject matter canceled herein.

Claims 1, 2, and 15 have been amended herein. Basis for the amendment may be found in the specification and claims as-filed. Thus, no new matter is presented by way of the present Amendment.

Applicants appreciate acknowledgement of the claim for priority as well as the acceptance of the new title, as set forth on page 2 of the outstanding Office Action.

Rejections under 35 U.S.C. 112

Claims 1, 2, 5 and 14-16 stand rejected under 35 U.S.C. § 112, second paragraph, as purportedly indefinite.

Claim 1 stands rejected as purportedly unclear with regard to the cell samples used in steps (a) and (b). Claim 1 is amended to recite a cell sample in step (a) as suggested by the Office.

Claims 1, 2, and 16 stand rejected as the Office argues that the action required to perform the steps was not recited. Claim 1 is amended herein to recite the additional steps. Applicants further provide the following comments.

The purpose of the first step recited in claim 1 is to establish the Panleucogate by identifying all the CD45+ expressing white blood cells and drawing a gate around these cells, eliminating debris or, if present, contaminated red blood cells. The purpose of the second step of claim 1 is to accurately identify the CD4+ lymphocytes and exclude other CD4+ expressing cells, by monocytes, from being falsely included in the secondary CD4+ lymphocyte gate. In previous methods, this was done by first ensuring that only CD4 expression of the CD45 bright expressing lymphocytes was analysed. This was made possible with the use of CD3, which further defined T cells within the bright CD45 "lymphoid" gate and ensured that the CD4+ expression analysed was only on T cells and not on monocytes.

In contrast, the presently claimed method uses neither of these previous methods to define CD4+ lymphocytes, but instead uses the relative differential expression of CD4 on lymphocytes and monocytes together with the relative differential side scatter expression on lymphocytes and monocytes to separate (and gate) CD4+ expressing lymphocytes (please refer to Figure 1b of the specification). The cells represented in this histogram are those identified in the reference population, *i.e.* the PanLeucogate. Gate "B" represents the identified CD4+ lymphocytes that have brighter CD4+ expression and less flow cytometric side scatter than monocytes which have dimmer CD4+ expression but more flow cytometric side scatter. Monocytes are then excluded from the reference population, *i.e.* the total white blood cell population or "panLeucogate". Thus, all relevant method steps are recited.

Claim 14 stands rejected for purportedly failing to define the method. Claim 15 stands rejected, as the Office states that the use of a hematology analyzer to determine the total white blood cell count is not a dual platform. Applicants provide the following comments as to claims 14-15.

The present invention may be used in either a dual platform or a single platform system. Although a haematology analyser itself is a single platform, it can be used together with another instrument, such as a flow cytometer, in a dual platform method. A single platform method is one of the methods claimed to perform the method (see Claim 1, 5 and 6). Single platform methodology is widely accepted as a reliable and reproducible method for CD4 lymphocyte enumeration (Barnett et al 1999: Absolute CD4+ T-lymphocyte and CD34+ stem cell counts by single-platform testing: the way forward: *British Journal of Haematology*: 1999, 106: 1059-1063, p1066, last paragraph in column 1). Although error may be introduced with single platform methodology that requires the pipetting of beads, which is an inherent problem with single platform testing, this does not mean that the invention cannot or should not be performed on a single platform.

Methods have been published to enable monitoring of the flow rate of the beads used in the single platform method on a flow cytometer and quality control and monitor for pipetting error. This is performed by calculating a bead flow rate (i.e. total number of beads counted in a single analysis divided by the time (in seconds)

of the analysis). This bead rate is relatively constant on the same flow cytometer because the beads are added in known volumes of known concentration. Thus, the bead rate calculated should remain constant over time. Pipette error (of beads or blood) will result in a different volume of the sample and will be different from the average bead rate. This is a useful means to monitor single platform analyses for pipetting error and is invaluable for improving quality control and avoiding the error that could be introduced by pipetting (referred to on pages 19-20 of the specification, as Scott et al, *Cytometry* 2005, 67B, see pg 31, col 2, lns 16 -23 and Col 3, 18-22 for details).

In light of the above amendments and remarks, Applicants request that the rejections under 35 U.S.C. § 112, second paragraph be withdrawn.

Rejections under 35 U.S.C. 102

Claims 1, 2, 15 and 16 stand rejected under 35 U.S.C. § 102(b) as purportedly anticipated by Melnicoff et al. (U.S. Patent No. 5,385,822), in light of Dorland's Illustrated Medical Dictionary, 2005. To anticipate a claimed invention under §102, a reference must teach each and every element of the claimed invention. *See Lindeman Machinenfabrik GmbH v. American Hoist and Derrick Company*, 221 USPQ 481, 485 (Fed. Cir. 1984). Applicants submit that Melnicoff fails to recite each element of the present invention.

Applicants submit that Melnicoff does not anticipate the present invention, as Melnicoff is actually directed to lymphocytes, and not to leukocytes. Applicants submit the following showing that Melnicoff recites "leukocytes" by mistake in example 3A. In any case, as leukocytes would not work in the context of Melnicoff, this cited reference is not enabled. "In determining that quantum of prior art disclosure which is necessary to declare an applicant's invention 'not novel' or 'anticipated' within section 102, the stated test is whether a reference contains an 'enabling disclosure'... ." *In re Hoeksema,* 399 F.2d 269, 158 USPQ 596 (CCPA 1968). The disclosure in an assertedly anticipating reference must provide an enabling disclosure of the desired subject matter; mere naming or description of the subject matter is insufficient, if it cannot be produced without undue experimentation.

Elan Pharm., Inc. v. Mayo Found. For Med. Educ. & Research, 346 F.3d 1051, 1054, 68 USPQ2d 1373, 1376 (Fed. Cir. 2003).

The following is provided to demonstrate that Melnicoff uses the word "lymphocytes". First, Applicants refer to specific sections of Melnicoff on behalf of the comments that follow:

1. Col 19, pnt 7, lns 51-52: *"Leukocyte counts were determined on a Coulter counter for each standard";*
2. Col 19, pnt 8, lns 53-55: *"Samples from each standard were aliquoted for immunofluorescence (for percent CD4 cells) and for use in the standard curve";*
3. Col 20 Pnt 16, lns 14-19: *"For immunofluorescence analysis, the sample of each cell standards from Step 8 (above) was labeled with FITC conjugated CD4 and PE conjugated CD45. The labeled cells were analysed by Flow Cytometry for* ***percent*** *of leukocytes (CD45+) which were CD4+lymphocytes";*
4. Col 20, Pnt 17, lns: 20-23: *"The number of CD4 cells in cell standard was calculated from the leukocyte and the percent CD4 lymphocytes measured by Flow Cytometry".*

The assignee on the face of Melnicoff (U.S. Patent No. 5,385,822) is Zynaxis. Reference is made at column 4, lines 35-40, to related patents/ or then co-pending applications and ongoing efforts by Zynaxis to develop a non-flow cytometric assay system for the determination of CD4 lymphocytes or other cell subpopulations, used for clinical decision making. Review of these co-pending and related patents, as well as correspondence with Dr. Melnicoff has revealed that U.S. Patent 5,385,822 was part of an ongoing project at Zynaxis extending from 1989 to 1993 and culminating in the release of the "Zymmune" assay. According to Dr. Melnicoff, this Zymmune assay is now no longer commercially available (per email communication with the present inventor, Dr. Glencross).

To this end, three specific patents with respect to the Zymune assay, in addition to US 5,383,822, are:

A. US 5,256,532, also to Melnicoff et al (application 345,436 listed as a co-pending application in US Patent 5,385,822, (col 3, lns 45 -61)) (attached);
B. Application 189,192, an abandoned Zynaxis application (see US 5,256,532 pg 1, Col 1, under "related US Application data, *...Continuation-in-part of Ser. No. 189,192 ...* ");
C. US 5,374,531, to Jensen et al, also assigned to Zynaxis.

Similar embodiments and examples are given throughout the patent specifications referred to above. In summary, they focus on descriptions of detectable reporter substances/ analytes (radio-isotope or fluorescent) which are uniformly incorporated into a cell sub-population/ subset of interest, methods of selective (affinity) cell separation (magnetic) to isolate the separated fraction of interest, and use of specific binding substances including a bio-membraneous form (bio-lipid) and related reagents. Systems to facilitate testing of increased volumes of tests with much shorter analysis times were also described relative to the flow cytometric standard. Although the initial related work did not appear to facilitate use of blood (with affinity separation methods were required) either in a solid (US 5,256,532) or fluid phase, subsequent improvements to the methodology allowed direct screening of complex biological fluids such as blood (US 5,385,822 and 5,374,531).

In all instances, there are examples of comparison of the above methodology to flow cytometric methods of cell enumeration, irrespective of phase of development (the prior art which was used as the reference standard for clinical decision making). Specifically, comment is made highlighting that the invention could be used as adjunct to or replacement for analytical techniques applied, e.g. flow cytometry (see US 5,385,822, col 5, lns 56-60; US 5,256,532 Col 6, lns 11-18; US 5,374,531, col 5, lns 24-31).

Evidence extracted from the latter mentioned patents is presented below to further clarify that the methodology of the flow cytometric method which was used in the comparative performance assessments/ examples is the same CD4 reference method used throughout, including Melnicoff (US 5,385,822):

- US 5,256,532 cited Landay and Muirhead (Clin Immunol Immunopath 52:48-60, 1989) as a reference in Col 1, lns 32-38 and on p2 col 1, lns 10-11, and referred to this reference in Example 2 (Col 20, lns 34-36, lns 54-58).
- US 5,385,822 also cited Landay and Muirhead and referred again to this reference in the text (Example 4, Col 22, lns 37-48, describes how the percent lymphocytes were derived. See also Col 6, lns 8-18 and another description in Example 3 (col 20, lns 15-25)).
- US 5,374,531 does not cite Landay and Muirhead but describes the reference method in Col 5, lns 42-54; Col 6, under "Description of the drawings", FIG 4, lns 54-59 and in Example 4, col 17, lns 7-11 and Col 18, ln 3-11.
- A peer reviewed publication by Denny et al, "Determination of CD4 and CD8 Lymphocyte Subsets by a New Alternative Fluorescence Immunoassay" (Clincal and Diagnostic Immunology, 1995, 2 (3), 330-336, p 334, col 2, lns 18-24) describes how the manufacturer (Zynaxis) used a Coulter instrument (as per description in US 5,385,822, col 19, lns 51) in the "reference method" to calibrate and evaluate the Zymmune methodology. Earlier in Denny it is stated that "the reference flow cytometric method" used in that report refers to the method by which the absolute cell concentration is calculated as the product of the absolute lymphocyte count and the relative subset level (percent) determined by Flow Cytometry (see p 331, Col 2, lns 30-35). Denny is especially important as it clearly outlines that a reference flow cytometric CD4 method was used by the manufacturer and concurs with the opinion that Melnicoff used Coulter counters in US 5,385,822. This is further supported by the fact that two of the co-authors of Denny were co-inventors listed on the face of Melnicoff (US 5,385,822).

Therefore, there appears to be consistency of methodology of the reference flow cytometric standard. In support that Melnicoff did use the standard of the time, in US 5,256,532 under "Summary of the invention", it is stated "..*The methods of the invention may be used as an adjunct to, and in certain instances as a replacement for the above noted analytical techniques currently applied in clinical laboratories, whose purpose is to screen for changes in cell frequency, e.g., flow cytometry.*"

Melnicoff et also state that they were making their system *"...applicable for clinical and diagnostic analysis of cell subsets of interest.."* (see Col 4 lns 35-40). It would therefore have been important for Melnicoff et al to compare their method to the standard of time, viz Flow Cytometry according to Landay and Muirhead, as they have cited under "other publications" (p2) and as they wrote themselves directly in the text (Example 4, Col 22, lns 37-48, describes how the percent lymphocytes were derived). Comparison to a non-standard method, i.e. using an alternative method to compare the performance of their alternative method, would be considered inappropriate to one versed in the art and is also as contrary to the intentions of Melnicoff et al as quoted above. It would therefore have been of no value to Melnicoff to perform a comparison as stated in example 3A in U.S. Patent No. 5,385,822 as, taken at face value, this was not made to the "analytical technique currently applied" (or standard of the time).

Melnicoff et al were measuring and identifying CD4+ cells within the population that were lymphocytes (CD45 +) by Flow Cytometry (Col 20, pnt 16, ln 19 and pnt 17, ln 22; col 21, pnt11, lns 35-36). "*The labelled cells were analysed by Flow Cytometry for the percent of leukocytes that were CD4+ lymphocytes*". Although the examiner has argued that Melnicoff should be interpreted as written, the applicant submits that this statement is ambiguous and could also be interpreted in the explanations that follow.

Applicants point out that CD4 itself cannot define lymphocytes alone but identifies both monocytes and CD4 expressing lymphocytes (see U.S. Patent No. 5,385,822, Col 22, lns 62 and 63). The only description of how CD4 percent was derived comes from Col 22, lns 37-48, where the method according to Landay and Muirhead is cited. CD45 was used in this context to primarily identify the lymphocytes that expressed CD4 and define the purity of the lymphoid gate. This can be interpreted from the wording "*...which were CD4 lymphocytes*" that follows the statement "*percent of leukocytes*". This can be taken to mean that the CD45 expression (specifically bright CD45+ expression) was used to identify and confirm the "lymphocyte" component which expressed CD4 to define the "percent of leukocytes". In other words, bright CD45 differential staining (which appears to have initially been derived from light scatter characteristics of lymphocytes, as per col 22,

In 44) was used to identify lymphocytes that expressed CD4 (*Col 20, Pnt 16, Ins 19*). CD45 bright differential staining would have been the obvious method of choice to one versed in the art to identify lymphocytes and the most effective means of identifying CD4+ lymphocytes in a 2-colour analysis (see Brando et al, 2000; p331, vol 1, "Precise Identification of Reference Populations", Ins 3-6; see also Barnett et al, 1997, p232, col 2, Para 4, In 12-14). CD45 staining was therefore crucial and especially relevant in the absence of other markers that would have been helpful in identification of CD4 lymphocytes, but not used by Melnicoff et al (e.g. CD3) in the described experiments. U.S. Patent No. 5,385,822 even refer to the use of differential CD45 staining to differentiate lymphocytes and monocytes (col 9, Ins 56-61). CD4+ lymphocytes can then be taken to mean "CD4 percent of lymphocytes".

Applicants submit that the statement used in U.S. Patent No. 5,385,822, "*percent of leukocytes*", is also ambiguous in this context (Example 3A, Col 20, Pnt 16, Ins 14-19 Pnt 17, 21-24, as well as Example 3C: Col 21, pnt11, Ins 35-40). The statement "*percent of leukocytes*" itself (col 20, pnt 16) could be taken to mean that reference was made to defining a percentage or sub-population of leukocytes, i.e. a white blood cell differential, a "percent...of leukocytes". This can also be better understood and interpreted in light of Dorland's Medical dictionary (cited by the Office) "Leukocyte count, differential - a leukocyte count that calculates the *percentages* of different types". Interpreted as such, it means that a percentage of the leukocyte population was and that the CD4 expressing cells were defined as a function of lymphocyte population and not the total leukocyte population.

This explanation, is consistent with terminology in the description of state of the art Flow Cytometry described in Col 6 In 14 of U.S. Patent No. 5,385,822, *i.e. "... X (percent lymphocytes in white blood cells) which also refers to the same population of cells as a "percent of leukocytes (CD45+)"*, (col 20, pnt 16). Further, this is confirmed by the text from Example 4, where direct reference is made to the publication cited by Melnicoff et al and co-authored by one of the inventors (Katherine Muirhead), where Melnicoff et al describes the exact method used to derive the percent of lymphocytes (including CD4, Col 22, Ins 11 and 40) according to the reference Landay and Muirhead (Clin Immun Immunopathol, 52: 48-60, 1989, Col 22, Ins 45 – 48). This text reads as follows: *[For measurement of percent*

labelled lymphocytes by Flow Cytometry, ... The percent lymphocytes labelled with each Mab was measured using standard analysis procedures, as described by Landay and Muirhead, supra.] See Landay and Muirhead, page 54, Fig 1: Validation of lymphocyte gates using FALS and log90° light scatter and demonstrating bright CD45 expression of the lymphocytes (Fig 1 legend, p 54, lns. 3-7) and the exclusion of monocytes (Fig1, A, B and C, p54). Also refer to Landay and Muirhead, page 57 ln 1-3 and lns 8-12 inclusive, on optimisation of a flow cytometric lymphoid gate for CD4 lymphocyte determination. A clear description of measurement of the CD4 percent (CD4+ expressing cells) that were lymphocytes is provided.

It is critical in dual platform CD4 lymphocyte enumeration that the population (CD4+ lymphocytes) identified by Flow Cytometry matches the population counted on a haematology analyser/ Coulter counter. This step will ensure that a correct count is calculated based on the same common cell population denominator identified between the two platforms in a dual platform system (see Brando et al, p330, Col. 1, lns 1-7). It would have therefore been important to ensure that the correct and same (common) denominator was used to ensure that the parameters used in the CD4 calculation were matched and the subsequent calculation correct.

As the "CD4+lymphocytes" which were a percent of leukocytes (CD45+) is cited as used to calculate the absolute CD4 lymphocyte count and as Melnicoff et al do not indicate *how* they separated out the CD4 lymphocytes within a population of all white blood cells by Flow Cytometry, it is likely that this would have resulted in an incorrect absolute CD4 count. U.S. Patent No. 5,385,822 suggests that this is further evidence that can be taken to mean that "the percent of leukocytes" was intended to define total lymphocytes. In the '822 patent, it is stated that if the calculation was performed exactly as written (Col 20, pnt 17, ln 21) using "leukocyte count" multiplied CD4+ lymphocytes" as defined in earlier steps (Col 20, pnt 16, lns 18-19), that parameters used in the calculation in Col 20, pnt 17, ln 21-22, are in fact mismatched (*i.e.* lymphocytes by Flow Cytometry and leukocytes by haematology analyser). This mismatch would result in the calculation of an incorrect absolute CD4 count. This can be attributed to the fact that the respective Flow Cytometry CD4 percent of lymphocytes and the "Leukocyte" reference population on the

haematology analyser do not represent the same population of cells, i.e. the denominator used on flow is a "percent of leukocytes" and taken to mean lymphocytes, whereas the population used on the Coulter counter was leukocytes (total). The applicant argues that it is unlikely that Melnicoff et al would have intentionally meant to wrongly calculate the absolute CD4 lymphocyte counts and that the correct denominator was in fact used, *i.e.* Total Lymphocyte count from the Coulter counter (the "percent of leukocytes" referred to above).

The Office states that Melnicoff et al may have used an alternative Flow Cytometry method, *i.e.*, "percent of leukocytes that were CD4+ lymphocytes" because of the inherent error associated with total lymphocyte count in the standard flow cytometric DP system, in order to get a better assessment of their new method, the applicant respectfully disagrees. Melnicoff cite this inherent lymphocyte error associated with standard Flow Cytometry to support the directness of their methodology described in the body of the specification (very similar passages of the advantages of their system are repeated throughout all related Melnicoff patents (see US 5,385,822, col 6, lns 1-18 and 27-56; US 5,374,531, col 5, lns 35-end and col 61-22). The strength of the Melnicoff method was that it did not use lymphocyte counts, or in fact total leukocyte, from a Coulter counter (see col 6, lns 1-4, col 6 lns 5-12, and col 18, lns 5—7) and was independent from haematology analysis.

Applicants also submit that U.S. Patent 5385,822 and related patents and publications (the "Zymune Assay") used the standard of the time. A further patent related to US 5,385,822 is US 5,256,532, also by Melnicoff et al, which states that the flow cytometric standard of the time was used (see Example 2 (Col 6 lns 26-37): "*Aliquots of cells from the same donor, which were not stained with PDCI, were immunofluorescence labeling. These samples were incubated with the same Mabs as above, then reacted with phycoerythrin conjugated goat anti-mouse immunoglobulin (Biomeda, Corp.). The cells were analyzed by flow immunofluorescence labeled cytometry, which is a method commonly used heretofore for this determination.*"

This flow cytometric standard is once again used in US 5,374,531 (col 4, lns 32-35 and col 5, lns 63-66). In all instances, the method of comparison and method used to determine the standardised curve of the Melnikoff method and to evaluate

the new method was the typical flow cytometric CD4 methodology described at the time based on the identification of lymphocytes. It would therefore appear that the same flow cytometric methods were used throughout the development of the method being developed by Zynaxis, ensuring continuity of the evaluation (by using the same standard for comparison, by dual platform lymphocyte based Flow Cytometry). Therefore, although it would appear from the misleading use of the word "leukocytes" that Melnicoff et al used an alternative method, the same Flow Cytometry standard was used throughout.

Thus, Applicants submit that there would be no purpose or value to determine and compare the usefulness of a new, alternative method to detect CD4 (the invention of U.S. Patent No. 5,385,822) by using an alternative Flow Cytometric method which would likely generate incorrect absolute CD4 counts. Use of an alternative flow cytometric method that would have likely generated an incorrect CD4 absolute count to evaluate a new method which was itself alternative, would not have been a good reference standard to compare one's new test.

Apart from the term "leukocyte" in example 3, other ambiguous statements are also used in the specification of the cited '822 patent.

The term "CD4 cells" indicates all leukocytes that express CD4, including CD4 lymphocytes as well as CD4+ monocytes, as reported by Melnicoff et al themselves (Col 15, lns 62-65 and Col 22, lns 62 and 63). This is different from the description in Col 20 (lns 20-24) and would indicate to one versed in the art that the description is used to delineate CD4+ lymphocytes and not CD4 cells. These terms are not consistent. As it is indicated by Melnicoff et al that they were identifying CD4 lymphocytes by Flow Cytometry, it is taken to mean that CD4 cells in this context means CD4 lymphocytes. Thus, terminology is loosely used in the specification of Melnicoff et al, including the use of the words "leukocyte" and "CD4". If, indeed, it was the intention of Melnicoff et al to count the total CD4 as indicated in example 3, pnt 8, ln 54, and use the leukocyte count (Col 19, pnt 7, lns 51-52) defined by Dorland's Medical dictionary, then Melnicoff was in fact calculating a total CD4 cell count which would have included monocytes. This is clearly not the intention of Melnicoff et al., as they repeatedly state that they were calculating CD4 lymphocyte count. Once again, this can be taken to mean that Melnicoff et al used the correct

denominator from the Coulter counter, ie., total lymphocyte count (which are not, incorrectly, also leukocytes).

In light of the above, Applicants submit that U.S. Patent No. 5,385,822 does not recite each and every element of the present invention, and request that the rejection under 35 U.S.C. § 102 be withdrawn.

Rejections under 35 U.S.C. 103

Claims 2, 5, 14 and 16 stand rejected under 35 U.S.C. § 103(a) as purportedly being unpatentable over Melnicoff et al. (U.S. Patent No. 5,385,822), in view of Brando et al. (Cytometry, 2000). To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. *See* MPEP § 2143.

As noted above, Melnicoff does not recite each element of the present invention. Brando et al. does not remedy the deficiencies of Melnicoff discussed above, as Brando is cited to show that it was known to use microbead-based technologies to count the number of leukocytes per volume of blood.

Claims 1, 2, 5 and 14-16 stand rejected under 35 U.S.C. § 103(a) as purportedly being unpatentable over Brando et al. (*Cytometry*, 1000), in view of Barnett et al. (*British Journal of Haematology*, 1999).

Applicants submit that Brando combined with Barnett do not provide motivation or expectation of success with the present invention. The benefits of the presently claimed methods are not disclosed or even suggested by the combined disclosures of Brando and Barnett.

The Office states that Brando discloses a method for enumeration of CD34+ cells which may be translated to be of use for CD4 lymphocyte enumeration, the benefits of use of total leucocytes as the denominator in the enumeration of CD4 lymphocytes is not evident or implied in their teachings, either alone or in

combination. However, Applicants submit that there is no reference in either of Barnett or Brando to indicate that use of leucocytes as a reference population for either dual or single platform CD4 enumeration would improve the outcome or reproducibility of CD4 lymphocyte measurements.

Firstly, Barnett repeatedly refers to methods to define lymphocytes for CD4 lymphocyte enumeration or identification by Flow Cytometry (see p 233, col 1, "T-Gate method"; p 234, col 2, "Lineage gating"; p235, col 2, "CD45/ SSC gating"; and p236 Col. 1, lns 8-11 and lns 15-16 and Figure 3, p 236) and do not mention total leucocytes or total CD45 expression in the definition of CD4 lymphocytes. Barnett et then teaches that CD4 lymphocytes are identified as CD3+CD4+ T lymphocytes refined with the secondary use of CD3 (see Figure 4, p237). Applicants note that these methods are different from the method in claim 1, which specifically avoids the use of CD3 and instead relies only on CD4 and side scatter.

Secondly, Barnett teaches that any abbreviated panel will result in loss of quality control checks (p237, col 1, lns 26-27). The method described in Claim 1 would be considered an abbreviated method in this context as it specifically deviates from the descriptions cited above (p233, 234 and 236), and deviates from the quality control taught by Barnett et al to correctly and accurately identify lymphocytes.

Finally, Brando and Barnett both teach away from the use of dual platform systems for CD4 lymphocyte enumeration that rely on methods that employ a haematology analyser to derive cell counts by calculation. For example, Barnett specifically states that both WCC and TLC are unreliable and therefore do not advocate any dual platform method, espousing single platform as the "way forward". In 1999, Barnett recommended Single Platform bead-based counting for CD4 enumeration and advised *against* use of dual platform methods that involve WCC (p 1059, col 1, ln 13-20), viz. in relation to lymphocyte subsets (inc. CD4) and CD34 counting, the following is stated: *"...The Dual Platform technique utlilises immunophenotypic data derived from the Flow Cytometer together with the total white cell count (WBC), or total absolute lymphocyte count (TLyC), obtained from a haematology analyser. It is recognised however, that a major factor contributing to the high inter-laboratory coefficients of variation reported for absolute CD4+ lymphocyte counts is the WBC generated by different haematology analysers...".*

This point is reiterated later in the paper (Barnett et al, 1999; p 1061, col 2, under DISCUSSION, ln 12): *"...The total WBC generated by haematology analysers has been recognised as a significant factor in the high percent CV for absolute counting CD4+ lymphocyte enumeration, a fact that has encouraged the development of Single platform approaches that use either bead or volumetric technology ....."*.

Brando also refers to the inaccuracy of the dual platform approach (Brando et al, p 330, col 1, lns 14-23), which to one of skill in the art would not likely encourage use of a WCC/ leukocyte count. Applicants especially point out these differences as compared to present claims 1 and 4, with regard to using a dual platform format.

Therefore, although the Office states that Barnett and Brando indirectly teach a method whereby total white blood cells could be used as a possible reference population for enumeration of CD4 lymphocytes, Brando and Barnett actually teach that it would be a disadvantage. Barnett cites that use of a WBC in fact represents a distinct disadvantage in the context of cell enumeration (see Barnett et al, 1999; p 1061, col 2, under DISCUSSION, ln 12 which begins "*...The total WBC ...*").

This method was discouraged and specifically taught away from by the cited references. The benefits were not known prior to the findings of the present invention.

In further support, Applicants refer to a publication after the initial publication of the present invention, the same group as that of Barnett (Storie et al) wrote in a single platform evaluation of leukocyte gating for CD4 enumeration, viz PanLeucogating, the following: *"...Recently a novel DP method has been described, termed PanLeucogating, that circumvents these problems (referring to use of WCC and differential counts) by being dependent on the WCC, a parameter more readily subjected to EQA and internal quality control than the WBC differential (meaning lymphocyte counts)."*

Further, the results of studies to prove benefit of the present invention are listed and referenced below to support the contention that the method of claim 1 would not have been obvious to one skilled in the art at the time of filing the patent application, in view of Brando and/or Barnett.

The advantages of using PLG CD4 gating using the WBC/ leucocyte count as the primary reference include the following.

Improved between- and within-laboratory reproducibility of dual platform methodology using total white blood cell gating/ PLG CD4 gating:

Both within- and between- laboratory performance/ reproducibility was improved using the gating described within Claim 1 in a dual platform system as evidenced by the data generated on the recent US National Institute of Health (Washington)-sponsored validation study of PLG at five United States laboratories. Here, a between-laboratory improvement of more than 33 percent and an improvement of within-laboratory reproducibility of at least 25 percent was noted using the methodology of the invention in a dual platform format (see abstract presented at the Congress on Retroviral and Opportunistic infections (CROI), held in Denver, NC in February 2006 (Addendum 1, CROI Abstract presented by Dr. T Denny in February 2006, see Conclusion, lns 29-31)). The data shows that the use of leukocytes as the reference for CD4 enumeration in a dual platform format revealed a one third improvement and a median "between-laboratory" percent CV of 9.3 percent . This between-laboratory percent CV is notably better, not only than traditional dual platform methods reported previously but is also better than percent CV reported for the currently advocated single platform methods (see Barnett et al, 1999, p1060, col 2 lns 11-14, w.r.t. CD4+ lymphocyte determination, *"...The inter-laboratory percent CV's were consistently lower for laboratories using the Single Platform approach (mean 13.7, range 10 – 18.3 percent) when compared to dual platform users (mean 23.4, range 14.5-43.7 percent)."* Therefore the method claimed in Claim 1 far exceeds traditional dual platform methods. In other words, the use of total leukocyte gating (referred to as PLG CD4 in the abstract) represents a significant improvement on between- and within-laboratory dual platform reproducibility.

Improvement using total white blood cell gating/ PLG CD4 method on single platform reproducibility:

The data to support markedly improved "between-laboratory" reproducibility between laboratories using the method of the present invention is shown in Addendum 2. The excellent results of the South African National Health Laboratory Service (NHLS) laboratories, which perform single platform (bead-based) total leukocyte gating methodology claimed in Claim 1 (PLG CD4) for CD4 lymphocyte counting, was presented at the XVI International AIDS meeting, held in Toronto in August 2006. This data has shown that the simplified 2-step gating strategy of the present invention can markedly improve reproducibility of single platform methodology (bead based) to derive CD4 lymphocyte counts. Hence, although the examiner has stated that per se, the use of beads for single platform cell counting is not novel, the benefit of improved between-laboratory reporting using the simplified gating method of the invention in a single platform format could also not have been anticipated by one skilled in the art.

Between-laboratory percent CV's of 7.0 and 8.5 percent were reported in this study for single platform-based leukocyte gating according to the present invention. The percent CV of 7 and 8.5 percent reported here far exceeds the between-laboratory percent CV for single platform methods reported by Barnett et al 1999 (p1060, col 2 lns 11-14), where a mean of 13.7 percent (range 10-18.3 percent) for the single platform approach was reported. The percent CV of 7 percent and 8.5 percent reported for laboratories that used the single platform simplified leukocyte gating method of the present invention was also notably better than the percent CV reported for other laboratories on the World Health Organisation CD4 EQAS programme (CV percent of 10.8 and 14.6 for the global World Health Organisation CD4 EQAS participants) or for QASI EQAS participants (CV percent of 10.6 and 15.8 percent, respectively). This represents between 50 percent and 100 percent improvement over traditional CD4 counting. An additional strength of the leukocyte gating strategy is that the between percent CV was maintained well less than 10 percent irrespective of analysis of lower CD4 counts, whereas other CD4 methodologies used by participants of both schemes did worse in the analysis of

lower CD4 counts (~250 cells/ul), i.e. percent CV of 14.6 and percent CV 15.8, respectively, for the WHO and the QASI international programme participants.

Increased window of PLG CD4 testing:

The method of the invention used in single platform format is not as affected by age as traditional (see 2005, HSRC Publishers, South Africa publication by Rehle T, Shisana O, Glencross DK and Colvin M. entitled "HIV-Positive Educators in South African Public Schools Predictions for Prophylaxis and Antiretroviral Therapy". ISBN 0-7969-2103-2). This study specifically required that samples be sent from surrounding rural areas to a centralised laboratory for CD4 testing to ensure that all educators across the region were accessed. For this reason, it was important to present supporting data of extended window of testing up to and including 5 days from venesection, to show that samples that took several days to get to the centralised laboratory would be of adequate quality for testing. This supporting evidence of delayed window of PLG concept testing in single platform format is included in Addendum 2 of this publication (see p40, "Supportive data from R&D, BCI, Miami, FL, USA" Forman M et al (see Background [paragraph] lns 6-7) and p42 (ln 5-7 and ln 15). Also Figure A8 (a) Single platform PLG CD4 aged sample analysis over 5 days and Figure A9 (p 42)). No significant differences of CD4 results were noted until 8 days ($P > 0.37$) on a Mixed model ANOVA analysis when either dual platform or single platform methods were used (($p=0.438$ and $p=0.157$ for SP and DP respectively) (see also p 42/43)). This finding was confirmed in another Beckman Coulter study performed in China on 30 samples where the Mixed model ANOVA analysis also revealed no statistically trend over time for single platform absolute CD4 counts, i.e. $p=0.8919$ (see p43, addendum 2, ISBN 0-7969-2103-2).

Similar data was also presented at the 2nd International AIDS conference on HIV pathogenesis and treatment, Paris, France in 2003 and published in Antiviral Therapy 8 (suppl 1), abstract number 1226 by Scott LE, Lawrie D and Glencross DK, entitled "CD4 monitoring in the developing world: A solution is PanLeucogating on aged samples". See addendum 3 below for details of abstract.

Less skill required to perform PLG CD4 testing

The method of the present invention is an easier alternative to standard gating, requiring less Flow Cytometry skills and can be taught more easily to technicians and technologists who would otherwise not be able to adequately perform more sophisticated traditional gating and Flow Cytometry. Testament to this is the excellent performance of laboratories using the method of the present invention to enumerate CD4 counts, and the ease that the methodology can be taught to laboratory personnel with no previous flow cytometry experience. See Addendum 2 abstract, paragraph CONCLUSION: *"Laboratories using PLG methodology have significantly outperformed both their African and global participants from resource-limited countries. NHLS/ARV laboratories exceeded the performance of the others by over 35 percent. This is a remarkable achievement considering that a vast majority of site personnel had limited flow cytometry skills..."*.

Cost savings

The method of the invention allows considerable savings that can have a marked impact on the rollout of antiretroviral treatment programmes, especially in cost-constrained countries. The Barbados government (Sippy et al; Comparison of the Panleucogating technique with four-colour heterogenous gating for CD4+ T cell enumeration by flow cytometry in HIV-1 infected individuals in Barbados) presented data at the XV International Aids meeting that was held in Bangkok in 2004 on validation data of both dual and single platform panleukogating according to the invention. They concluded that use of the single or dual platform method can facilitate marked savings for their national antiretroviral treatment programmes (see conclusion of the poster presented). Similar concluding remarks were made by the CDC group in Uganda on savings with use of dual platform PanLeucogating in data presented as a poster at the XIV International AIDS conference held in Barcelona (see Conclusion paragraph (lns 9-15) of the poster Abstract MoPeB3104, in 2003, also see lns 7-10 of the abstract).

Data on the savings to South Africa over ten years was also presented at the 2nd South African AIDS meeting held in July 2005. A further publication from Thailand (see ABSTRACT from Cytometry Part B, 65B, 29-36) also concludes (under Conclusions): *These observations demonstrate that CD4 enumeration by*

PanLeucogtaing is reliable and can be performed to an identical standard by a quality-assured network of collaborating laboratories as a new cost effective approach to HIV monitoring." (See also Cytometry Part B, 65B, 29-36, and Pg 36, col 1, Ins 18-30 which reads "*The results of this study demonstrate that the PanLeucogating strategy is a robust reliable method for CD4 T–cell enumeration*". *The adoption of this method and the use of generic reagents can decrease the cost of CD4 testing from $12 to $2,50 per test. The Thai government has declared that the 10 000 HIV infected patients will be given access to antiretorviral therapy by 2005. It is estimated that 150 000 to 200 000 CD4 assays per year will required and that the number of tests per year will increase as more HIV patients gain access to antiretroviral therapy. The PanLeucogating protocol and the use of generic reagents represent an affordable alternative to CD4 enumeration in resource-poor settings. In South Africa, similar savings have been realised with use of both single and dual platform PanLeucogating methods"* (see The Southern African Journal of HIV Medicine, September 2005, p 38-41, specifically see p 40, col , Ins 39-44). Details of this presented data are shown in Table 1 attached hereto.

In light of the above, Applicants request that the rejections under 35 U.S.C. § 103 be withdrawn.

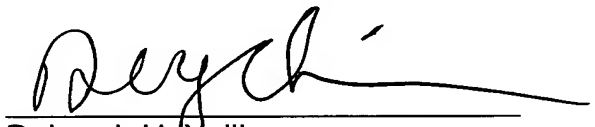
CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this Reply or the application in general, it would be appreciated if the Examiner would telephone the undersigned attorney so that prosecution of this application may be expedited.

Respectfully submitted,

BUCHANAN INGERSOLL & ROONEY PC

Date: October 23, 2006 By: 

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